Journal of Medical Virology 54:192–195 (1998)

HPV 16 E7 Antibody Levels in Cervical Cancer Patients: Before and After Treatment

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Antibody response to HPV16 E7 oncoprotein may represent a marker of cervical cancer. A HPV16 GST-E7 fusion protein was used in a Western Blot assay to analyse the HPV16 E7 antibody response in 30 patients before and after treatment for cervical carcinoma (stage IIB or IIIB). Patients were treated with three courses of cisplatin/bleomycin therapy followed by surgery, or with surgery alone. Thirteen out of 30 patients had serum antibodies to HPV16 E7 antigen. Three months after chemotherapy little or no change in antibody titre was detected. In contrast, after surgery, a significant decrease in antibody titre was observed in 9/10 patients. In two cases the titre declined to zero 3 and 9 months after treatment, respectively. These results confirm the usefulness of studying anti-HPV16 E7 antibody profile in cervical cancer patients and suggest that the serum response correlates with tumour burden. ***J. Med. Virol. 54:192–195, 1998.***

KEY WORDS: Human Papillomavirus type 16; anti-E7 IgG titre; cervix cancer; therapy

INTRODUCTION

Infection by "high risk" Human Papillomavirus (HPV) has been recognised to be the major risk factor for cervical cancer development [Munoz et al., 1992; Bosch et al., 1995]. HPV 16 is the predominant type associated with this tumour, and its DNA is found often integrated into the cellular genome. The E6 and E7 genes, responsible for cellular transformation, are consistently retained in cervical cancer cells and actively expressed. The HPV16 E7 gene product, a small nuclear phosphoprotein, is the major transforming protein of the virus and the most abundant viral protein in cervical neoplasia [Stoler et al., 1992].

Diagnosis of HPV 16 infection has relied on the demonstration of HPV DNA in clinical samples by nucleic acid hybridisation or gene amplification techniques. Serological assays for HPV16 have been hampered by the lack of available antigen source, but in recent years great improvement has been achieved. Several groups have investigated the serological response to the virus antigens in HPV16 induced diseases using different antigen sources such as recombinant proteins expressed in prokaryotic or eukaryotic systems, or synthetic peptides [Galloway, 1996; Kirnbauer et al., 1994]. Despite the variety of techniques used and of the patient populations examined, a higher antibody response to HPV16 E7 protein has been detected consistently in cervical carcinoma patients than in clinically normal controls or in patients with preneoplastic lesions [Jochmus-Kudielka et al., 1989; Muller et al., 1992; Di Lonardo et al., 1994]. These data suggest that a humoral response is mounted when invasiveness has been established. In addition, a strong correlation has been reported between seropositivity to HPV16 E7 and tumour stage [Fisher et al., 1996]. Therefore, concordant results indicate that antibodies to HPV16 E7 protein may be as useful markers for HPV-associated cervical carcinoma as the presence of HPV16 DNA.

To date, few data are available about the effect of treatment on the humoral response to HPV 16 E7. A decrease or a disappearance of antibodies to HPV16 E7 has been reported by some authors [Fisher et al., 1996; Dillner, 1990; Baay et al., 1995]. Given the possible value that changes in HPV antibodies may have in predicting disease development, recurrence and treatment response, these preliminary observations need confirmation. We therefore evaluated the antibody response to HPV 16 E7 in 30 women with cervical cancer before and after treatment.

MATERIALS AND METHODS

Patients and Clinical Specimens

Sera were obtained from 30 patients (median age 46.3 years) attending the Department of Obstetrics and Gynaecology, Catholic University of Rome. All patients had squamous cervical carcinoma and were staged as

Contract grant sponsors: AIRC, CNR/ACRO, and the Italian Ministry of Public Health.

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Accepted 15 October 1997

IIB or IIIB according to the recommendations of the International Federation of Gynaecology and Obstetrics (FIGO). Patients were treated by neoadjuvant chemotherapy consisting of two or three courses of cisplatin (40 mg/m^2, Day 1–4) and bleomycin (15 mg/m^2, day 1,8) with 3 week interval between cycles. After chemotherapy, surgical treatment was undertaken for those patients whose tumour size was decreased under 4 cm and parametrial and vaginal disease were judged resectable by radical operation. Interval time between the completion of chemotherapy and surgical treatment was 1–3 months. Three patients with tumour burden less than 4 cm received only surgical treatment. Sera collected before treatment and after chemotherapy and/or surgery were available. An informed consent was obtained from all patients participating in this study.

Expression and Purification of GST-E7 Fusion Protein

The HPV16 E7 ORF was amplified from recombinant pBR322-HPV16 plasmid (a gift from Dr. E.M. De Villiers) by PCR with primers containing EcoRI and XhoI tails for directional cloning into the prokaryotic expression vector pGEX-4T1 (Pharmacia Biotech, Uppsala, Sweden). The recombinant plasmid GST-E7 HPV16, containing the pTac promoter and the fusion gene coding for Glutathione-S-transferase and HPV16 E7 protein (GST-E7), was transfected in E.Coli XL1 blue and protein expression was induced by addition of isopropylthio-β-galactoside to final concentration of 0.1mM and incubation for 4 hours at 30°C. The bacteria were resuspended in cold phosphate saline buffer (PBS) and sonicated; cellular lysis was completed by gentle mixing with Triton X-100 at 1% final concentration. Solubilized proteins were spun down by centrifugation at 12,000 rpm and GST fusion proteins purified by affinity chromatography on a glutathione sepharose 4B column. GST fusion proteins were eluted by three washes with buffer (10 mM glutathione, 50 mM Tris.HCl pH 8.0). Separation of GST-E7 fusion proteins from residual bacterial contaminants was carried out by centrifugation in Microcon microconcentrator tubes (Amicon Inc, Beverly, MA). The protein concentration was measured by reaction with the GST substrate 1-chloro,2,4,-dinitrobenzene by a commercial assay (Pharmacia Biotech, Uppsala, Sweden).

Western Blot Analysis

GST-E7 fusion protein (100 ng/lane) was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane in 48 mM Tris pH 9.2, 39 mM glycine, 1.3 mM SDS, and 20% methanol by a semidry blotting apparatus. Filters were blocked in buffer (0.05M Tris-HCl pH 7.2, 1M NaCl, 0.1% Tween 20) for 3 hours at room temperature and cut into strips of 0.5 cm length. Human sera dilutions ranging from 1:100 to 1:1,400 were incubated with the strips at 4°C overnight. Sera collected at different times from the same patient all were assayed in the same experiment and tested twice with the same E7 antigen preparation. Strips were left to react with a 1:1,500 dilution of rabbit anti-human IgG and then with a 1:1,000 dilution of biotinylated $F(ab')_2$ fragment of swine anti-rabbit (Dakopatts, Glostrup, Denmark). Finally, the strips were incubated with streptavidin-biotinylated horseradish complex for 30 minutes. Each reaction was followed by two washes in blocking buffer. Bound human antibodies were visualised by staining with Diaminobenzidine (DAB). The positive band at the limit dilution was detected by densitometric scanning with a Mustek MFS-6000CX apparatus and data were analysed using Phoretix 1D software. To exclude any reactivity between human sera and GST protein, each serum was also tested with a strip containing only GST protein.

RESULTS

The recombinant E7 protein of HPV-16 fused with glutathione S-transferase from Schistosoma Japonicum (GST) at its carboxyl terminus was used as antigen in serological analysis. This fusion protein can be recognised in Western blot by a commercial anti-HPV16 E7 monoclonal antibody (Triton Diagnostic, Alameda, CA), indicating that at least one E7 specific epitope is present in the fused protein (data not shown).

Samples from 30 women with cervical carcinoma (stage IIB and IIIB) were tested simultaneously for the presence of serum antibodies to HPV16 E7 antigen and for reactivity against GST protein alone by Western Blot. No sera showed reactivity with GST protein. An example of Western blot analysis is shown in Figure 1.

Thirteen out of 30 patients had antibodies to HPV16 E7 protein with a prevalent IgG titre of 1:1,000, whereas the other patients showed no serological response at any time during the study (Table I).

After completion of chemotherapy, no difference in seroreactivity was observed between pretreatment and postchemotherapy samples with the exception of case 8,640 that revealed a small decrease in IgG titre after chemotherapy (Table I).

Analysis of postsurgery sera showed a decreased seroreactivity to E7 antigen, irrespective of tumour stage and previous chemotherapy. The antibody decline was observed 1 month after surgery in five cases, and 3 months later in four cases. Two patients became seronegative after surgery; case 7,217 with a low level of anti-E7 antibody before treatment had no detectable anti-E7 antibodies 3 months after surgery (Table I) whereas patient 2,043 became seronegative 6 months later (Fig. 1 and Table I). This woman was the only patient whose serum was collected 9 months after surgery.

DISCUSSION

Serum samples from 30 women with advanced squamous cervical carcinoma were studied to investigate the possible influence of treatment on antibody re-

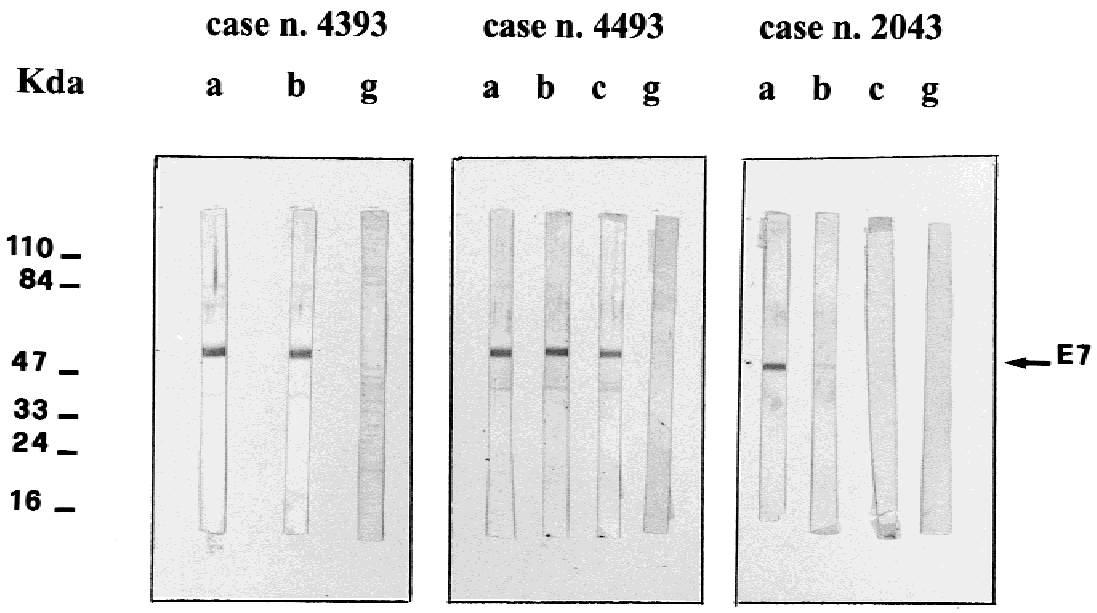

Fig. 1. Western blot analysis of sera from patients with cervical cancer before and after treatment. GSTE7-HPV16 fusion protein and GST protein were transferred to nitrocellulose membranes and strips were incubated with 1:200 dilution of human sera. Positive reaction was detected with biotinylated anti-human IgG followed by incubation with streptavidin-biotin-peroxidase complex (Vector, Burlingame,CA). Staining was performed with DAB. Case number 4393: Patient treated with chemotherapy only. Case number 4493: Patient treated with surgery only. Case number 2043: Patient treated with chemotherapy followed by surgery. **lane a:** Before treatment; **lane b:** after treatment (4493 and 2043, 1 month after surgery); **lane c:** after treatment (4493, 3 months after surgery; 2043, 9 months after surgery); **lane g:** strip with GST protein alone incubated with pretreatment serum.

sponse to HPV16 E7 antigen in cervix cancer patients. The sera were analysed for changes in antibody levels before and after treatment using a GST-E7 fusion protein in Western blots. The use of densitometric scanning to determine the sera limit dilution permits a good reproducibility and accuracy, allowing to detect minimal changes in serum titre as in the cases 8,640 and 4,493.

Antibodies to HPV 16 E7 protein were detected with titres of 1:1,000 in a high proportion of patients (13 of 30). These data confirm the association of anti-HPV16 E7 IgG with cervical cancers [Jochmus-Kudielka et al., 1989; Muller et al., 1992; Di Lonardo et al., 1994]. The remaining 17 patients with no detectable response before treatment never produced anti-E7 antibodies. Lack of seroreactivity is still unclear and it could be due to either a failure of the immune system, an immunotolerant status or an infection by different HPV types.

In women with an anti-E7 humoral response, we observed a significant decline of anti-E7 IgG titre only after surgical treatment, irrespective of tumour stage. Conversion to seronegative status was detected in two cases. Women with lower pretreatment anti-E7 IgG titres become seronegative earlier than women with high titres, suggesting an inverse relationship between anti-E7 antibody titre and time required for attaining seronegativity. No evidence of disease was detected in any of the patients after completion of chemotherapy and surgical treatment. Dillner [1993] and more recently Fisher et al. [1996] reported a disappearance or a decrease of antibodies to HPV16 E7 in cervical cancer patients after radiotherapy alone. Baay et al. [1995] investigated the antibody response to HPV16 E7 in a follow-up study of women treated by radiotherapy and/or surgery. Interestingly, and in agreement with our results, decrease of anti-E7 IgG titre or seronegativity was demonstrated in patients with clinical remission, while progression was accompanied by unchanged or increased anti E7 IgG titre.

The results confirm the effect of treatment on anti-E7 IgG levels. Data from our patients suggest that the decline in antibody titre follows surgical treatment, as anti-E7 IgG titres were lower in postsurgery than in postchemotherapy samples, whether or not surgery was preceded by chemotherapy.

It cannot be excluded that antibody titres might decrease with time postchemotherapy. However sera were collected at the same time point (3 months) after surgery or after chemotherapy ruling out that sampling time had affected antibody titres.

Although chemotherapy was effective in reducing the tumour burden to a resectable size (<4 cm) in almost all patients (data not shown), this reduction did not produce changes in the anti-E7 antibody titre, with the exception of case 8640. This suggests that removal of tumour rather than size reduction influences the antibody response to E7 protein.

In conclusion, the results from the present study suggest that a drastic reduction in tumour burden is correlated with anti-E7 antibody decrease. Further investigations are needed to assess the prognostic signifi-

TABLE I. Anti HPV16-E7 IgG Titre Before and After Treatment in Cervical Cancer Patients*

			Time of sera collection			
Case	Stage	Therapy	A	B	C	D
8,387	IIB	CT/S	1:200	1:200	1:400	1:200
6,135	IIB	CT/S	neg	neg	neg	nd
312	IIIB	CT/S	nd	1:1,200	1:1,200	1:200
4,393	IIB	CT	1:1,000	1:1,000	nd	nd
5,743	IIB	CT/S	neg	neg	neg	nd
3,624	IIIB	CT/S	neg	neg	neg	nd
7,217	IIB	CT/S	1:400	1:400	1:200	neg
8,109	IIB	S	1:1,200	nd	1:1,200	1:400
2,770	IIIB	CT	neg	neg	nd	nd
8,640	IIB	CT/S	1:1,000	1:800	1:200	nd
5,587	IIIB	CT/S	neg	neg	neg	nd
4,472	IIB	CT/S	neg	neg	neg	nd
9,017	IIIB	CT/S	nd	1:1,200	1:400	1:800
7,673	IIB	CT/S	neg	neg	neg	nd
7,577	IIIB	CT	1:1,000	1:1,000	nd	nd
359	IIB	CT/S	nd	neg	neg	neg
2,043	IIB	CT/S	1:1,000	1:1,000	1:200	neg[a]
150	IIB	CT/S	nd	neg	neg	neg
8,797	IIIB	CT/S	neg	neg	neg	neg
7,773	IIIB	CT/S	1:1,200	1:1,200	1:800	1:400
8,361	IIB	CT/S	nd	neg	neg	neg
7,425	IIIB	CT/S	neg	neg	neg	nd
1,739	IIB	S	1:1,000	nd	1:1,000	1:400
9,046	IIIB	CT/S	neg	neg	neg	nd
1,859	IIB	CT/S	neg	neg	neg	nd
2,155	IIIB	CT	1:1,000	1:1,000	nd	nd
1,323	IIB	CT/S	neg	neg	neg	nd
6,570	IIIB	CT/S	neg	nd	neg	neg
4,493	IIB	S	1:1,000	nd	1:1,000	1:800
3,318	IIB	CT	neg	neg	nd	nd

*nd = not done; CT = chemotherapy; S = surgery; CT/S = chemotherapy and surgery; A = beginning therapy; B = 1–3 months after the completion of chemotherapy; C = 1 month after surgery; D = 3 months after surgery.
[a]Nine months after surgery.

cance of anti HPV-16 E7 titre in the management of cervical cancer patients.

ACKNOWLEDGMENTS

This study was carried out at Laboratory of Virology, Regina Elena Institute for Cancer Research, Rome, Italy. The authors thank Prof. MS Campo (Beatson Institute for Cancer Research, Glasgow) for the critical reading of the manuscript and Dr. G. Scambia (Dept. of Ob/Gyn, Catholic University Rome) for providing serum samples and clinical data.

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